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Stoichiometry of Monoclonal Antibody Neutralization of T-Cell Line-Adapted Human Immunodeficiency Virus Type 1

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In order to study the stoichiometry of monoclonal antibody (MAb) neutralization of T-cell line-adapted human immunodeficiency virus type 1 (HIV-1) in antibody excess and under equilibrium conditions, we exploited the ability of HIV-1 to generate mixed oligomers when different *env* genes are coexpressed. By the coexpression of Env glycoproteins that either can or cannot bind a neutralizing MAb in an *env* transcomplementation assay, virions were generated in which the proportion of MAb binding sites could be regulated. As the proportion of MAb binding sites in Env chimeric virus increased, MAb neutralization gradually increased. Virus neutralization by virion aggregation was minimal, as MAb binding to HIV-1 Env did not interfere with an AMLV Env-mediated infection by HIV-1(AMLV/HIV-1) pseudotypes of CD4$^-$ HEK293 cells. MAb neutralization of chimeric virions could be described as a third-order function of the proportion of Env antigen refractory to MAb binding. This scenario is consistent with the Env oligomer constituting the minimal functional unit and neutralization occurring incrementally as each Env oligomer binds MAb. Alternatively, the data could be fit to a sigmoid function. Thus, these data could not exclude the existence of a threshold for neutralization. However, results from MAb neutralization of chimeric virus containing wild-type Env and Env defective in CD4 binding was readily explained by a model of incremental MAb neutralization. In summary, the data indicate that MAb neutralization of T-cell line-adapted HIV-1 is incremental rather than all or none and that each MAb binding an Env oligomer reduces the likelihood of infection.

The prospects of developing an effective vaccine based on humoral immunity against a viral infection may depend on the stoichiometry of antibody-mediated virus neutralization. For poliovirus, for which an antibody-inducing vaccine is protective, it has been reported that virus neutralization can be accomplished by the binding of four monoclonal antibodies (MAbs) to a virion (16). In this case, virus capsid can exist in two different conformations—infectious and noninfectious—with different electrophoretic behavior, and bivalent binding of a single or few antibodies locks the conformation of the capsid in the noninfectious conformation (12, 20). Similarly, adenovirus, for which humoral immunity is highly protective, may be neutralized by the binding of a single antihexon antibody molecule (39). Binding of antihexon antibodies seems to block a conformational change normally induced in an acidic environment (39). In the case of human immunodeficiency virus (HIV), subunit vaccines only inefficiently elicit neutralizing antibodies (21) and have shown limited protection in vaccination trials (1a, 5). If HIV proves inherently difficult to neutralize compared to other viruses for which effective vaccines are available, this could help explain the relative failure of HIV subunit vaccine candidates and provide a scientific foundation to evaluate antibody-based strategies for HIV vaccine development.

The envelope glycoprotein (Env) of HIV promotes attachment and fusion with permissive cells and is a target for virus-neutralizing antibodies. The Env glycoprotein is synthesized as a precursor, gp160, which oligomerizes upon folding within the endoplasmic reticulum (ER) (11) and is subsequently proteolytically cleaved in Golgi to gp120, the surface protein of HIV type 1 (HIV-1), and to gp41, the transmembrane protein of HIV-1. The assembly domain responsible for Env oligomerization is located in extracellular gp41 (10). This domain is functionally conserved among HIV and simian immunodeficiency virus (SIV) strains; thus, HIV-1 is capable of forming mixed Env oligomers with HIV-2 and SIV when coexpressed in the same cells (7). Structural data on gp41 strongly suggest that HIV Envelope oligomers are trimeric (3, 38). The formation of mixed oligomers between related Env species probably occur by the random recruitment of monomeric subunits from a common pool in the ER, as has been shown for the formation of mixed influenza hemagglutinin trimers (2).

Antibody neutralization of animal viruses has often been studied by determining the kinetics of antibody neutralization (16, 22, 36, 39), and the presence of first-order kinetics without a lag phase has often been interpreted as an indication of the presence of a single-hit mechanism of action of antibody neutralization (8). An initial lag phase indicating a multihit mechanism of neutralization may, however, be obscured by the rapidity of the antigen-antibody reaction (6, 8). Thus, complicated determination of the amount of antibody bound per virion is often necessary (16, 36, 39). For antihexon antibody-neutralizing adenovirus, a single bound antibody results in neutralization (39). In other cases, discrepancies between apparent first-order kinetics of neutralization and the amount of antibody bound to virus to accomplish neutralization have been explained by the hypothesis that only a minority of the antibody-binding sites are critical for neutralization (16, 36). First-order kinetics of MAb neutralization of HIV-1 have been demonstrated (22). However, as pointed out by Icenogle et al., (16), in addition to a single-hit action of the neutralizing antibody, neutralization kinetics that approximate first order may also be explained by incremental neutralization, i.e., each antibody binding decreases the infectivity of the virion by a fraction. In an effort to determine which of these two different

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mechanisms of MAb neutralization is correct, we exploited the ability of HIV-1 to generate mixed Env oligomers when different envelope genes are expressed within the same cell. By the coexpression of two envelope genes encoding Env proteins either binding or resistant to binding of a neutralizing MAb in an Envelope transcomplementation assay (15), virions were generated in which the proportion of MAb binding sites to Envelope protein present could be regulated. Thus, the amount of antibody bound to virus could be controlled in mixtures of virus and excess antibody under equilibrium conditions. By the study of the sensitivity to MAb neutralization of such virions, the amount of antibody binding to Envelope required for neutralization may be determined and different theories regarding antibody neutralization can be experimentally addressed.

MATERIALS AND METHODS

HIV expression vectors. pSVIIIenv, which expresses *rev* and *env* under control of HIV-1 long terminal repeat, and pHxBΔenvCAT, an *env*-defective HIV-expression vector containing a chloramphenicol acetyltransferase (CAT) gene in the *nef* open reading frame of HIV-1, were kindly donated by Joseph Sodroski (15). The following plasmids were derived from pSVIIIenv by the substitution of a 2.1-kb *Kpn*I-*Bam*HI fragment with a corresponding fragment of BRU *env*: pSV-A308, pSV-A308T321, and pSV-A308K373. pSV-A308 contains a T308A mutation within the V3 region of gp120, disrupting a site for N-linked glycosylation at position 306 (14); pSV-A308T321 contains an additional A321T mutation at the tip of the V3 loop, rendering A308T321 gp120 resistant to MAb binding in this region (31). pSV-A308K373 contains in addition to the T308A mutation a D373K mutation, which renders A308K373 gp120 defective in CD4 binding (25). An *env*-defective derivative of pSVIIIenv, pSV-dBgl, containing an 580-bp out-of-frame deletion within the *env* gene, was used as a mock plasmid to determine background CAT activity in the neutralization assay.

A plasmid expressing envelope of amphotropic murine leukemia virus (AMLV), pSV-aMLVenv, was kindly donated by A. Panganiban.

Antibody affinity determination by enzyme-linked immunosorbent assay (ELISA). This was done by using the method of Moore et al. (23) exactly as previously described (31).

Virus generated by *env* trans-complementation. HIV-1 Env chimeric virus was generated by cotransfecting HEK293 cells (13) with mixtures of *env*-expressing plasmids and pHxBΔenvCAT. Briefly, 293 cells were seeded in 6-well microculture plates (NUNC) the day before transfection at a density of 5×10^5 cells per well. Cells were then transfected with 4 μg of *env*-expressing plasmid and 5 μg of pHxBΔenvCAT by the calcium phosphate precipitation method. Twenty-four hours posttransfection culture medium was changed, and after an additional 48 h, the virus-containing supernatant was harvested and cleared by filtration (0.45-μm-pore-size filter).

For the generation of HIV-1 pseudotypes incorporating (AMLV), COS-1 cells were cotransfected with pHxBΔenvCAT, pSV-A308, and pSV-aMLVenv by lipofection with Lipofectamin Plus reagent (Gibco) according to the instructions of the manufacturer.

Virus neutralization assay. HeLa-CD4 clone 1022 cells (4) were plated the day before inoculation in 24-well microtiter plates (NUNC) at a density of 10^5 cells per well. Virus containing supernatants produced by envelope transcomplementation were divided in two aliquots and incubated for 30 min at room temperature either in the absence or presence of 2 μg of V3-directed MAb NEA-9205 (Dupont NEN)/ml (9) before being transferred to the HeLa-CD4 cells. Medium was changed 24 h postinoculation, and the cells were incubated an additional 48 h. Then cells were washed once in PBS and lysed in 300 μl of Reporter Lysis buffer (Promega) and assayed for CAT expression as previously described (19). Assay background was determined from a pSV-dBgl-complemented supernatant processed in parallel.

Only freshly prepared virus supernatants were used for the neutralization assay, as preparations of chimeric virus that had undergone freezing and thawing proved more sensitive to neutralization than freshly prepared supernatants.

Western blot analysis of HIV-1 Envelope expression. For the analysis of HIV-1 expression, 293 cells were transfected as described above. Three days posttransfection, the supernatant was harvested and cleared by filtration (0.45-μm-pore-size filter). The cells were washed once in phosphate-buffered saline and then lysed in 300 μl of cold lysis buffer (1% Triton X-100, 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% albumin, 100 IU of aprotinin/ml. Insoluble material was cleared from the cell lysates by centrifugation.

Virions present in the supernatants harvested were separated from soluble protein by centrifugation (15,000 × *g* for 90 min; 4°C) as previously described (30). An infectious HIV-A308-containing supernatant propagated in H9 cells and generated as previously described (14) was processed in parallel. Virion pellets were prepared from 600 μl of supernatant generated by envelope transcomplementation and from 300 μl of virus supernatant propagated in H9 cells.

Virion pellets were lysed in 1× Novex LDS sample buffer containing 1× reducing agent (Novex). Cell lysates and virion-depleted supernatants were adjusted to contain 1× LDS sample buffer and reducing agent before all samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on precast 4 to 12% Bis-Tris gels (Novex) and subsequently transferred to polyvinylidene difluoride membranes. Env proteins were detected with both P4D10 (kindly donated by L. Åkerblom) (1) and gp-serum B, a guinea pig immune serum (kindly donated by A. Bolmstedt) raised against recombinant HIV-BRU gp120 and previously described in detail (31). Gag proteins were detected by using BC1071, a mouse anti-p24 MAb commercially available from Aalto BioReagents. Binding of primary antibody was detected by chemiluminescence with the WesternBreeze Mouse chemiluminescence detection kit (Novex) according to the instructions of the manufacturer, except that the secondary antibody solution for detection of guinea pig immunoglobulin (Ig) was added a 1:2,000 dilution of an alkaline phosphatase-conjugated anti-guinea pig Ig antibody (Sigma A-5062).

Mathematical analysis. Data from neutralization of chimeric virions were analyzed by nonlinear regression with Prism software, version 2.01 from GraphPad. For analysis, each value of V/V_0 was treated as a separate replicate. The functions that were fit to the experimental data are described in the text. The applicability of each function fit to the experimental data was evaluated by calculating R^2 and using the runs test for goodness-of-fit.

RESULTS

MAb binding to A308 and A308T321 Envelopes. Variants of HIV-BRU lacking the N306-glycan at the base of V3 loop, as HIV-A308, are efficiently neutralized by a V3-directed MAb (for HIV-A308, the 50% inhibitory concentration [IC_{50}] = 0.0043 μg/ml) (14, 30). Accordingly, the V3 loop of virion-associated A308-Envelope is readily accessible to MAbs, in contrast to virion-associated Envelope containing the N306-glycan (30). A MAb-selected variant of HIV-A308, HIV-A308T321, totally resists neutralization by NEA-9205 (IC_{50} > 5 μg/ml) (31). This variant also lacks the N306-glycan shielding the V3 loop on virion-associated Envelope. To determine whether neutralization resistance of HIV-A308T321 was accompanied by a resistance to MAb binding, ELISA affinity determinations of NEA-9205 binding to HIV-A308 and HIV-A308T321 gp120 were done (Fig. 1). This showed that A308T321 gp120 did not bind NEA-9205 at concentrations up to 2.5 μg/ml. In contrast, A308-gp120 bound NEA-9205 with high affinity. Thus, chimeric virions generated by coexpression of A308 *env* and A308T321 *env* may be expected to bind NEA-9205 in proportion to their A308 Envelope content.

Coexpression of envelope genes. To determine the ratio between A308 and A308T321 Envelope when *env* genes were coexpressed, pSVIII-A308 and pSVIII-A308T321 and an equal mixture of the two were cotransfected with pHxbΔCat in HEK293 cells. Virions present in the cell supernatant were gently pelleted by centrifugation, and cell lysates, virion-depleted supernatants, and virion pellets were subjected to immunoblotting by using MAb P4D10 and immune guinea pig serum B (gp-serum B) raised against HIV-BRU gp120 as previously described (31). As seen in Fig. 2, MAb P4D10 was specific for A308 Envelope and unreactive with A308T321 Envelope. In contrast, gp-serum B displays equally high affinities for native A308- and A308T321 Envelope (31) and is reactive with both Envelopes by an immunoblotting procedure (Fig. 2). Thus, the amount of A308 Envelope expressed may be estimated by using MAb P4D10, and the total amount of Envelope present may be estimated by using gp-serum B. When equal amounts of pSVIII-A308 and pSVIII-A308T321 plasmids were cotransfected, the ratio between P4D10- and gp-serum B signal in cell lysates, virion-depleted supernatants, and virion pellets was intermediate between the ratios obtained with pure pSVIII-A308 transfections and pure pSVIII-A308T321 transfections (Fig. 2). Thus, the ratio between A308 and A308T321 Envelope proteins incorporated into virions

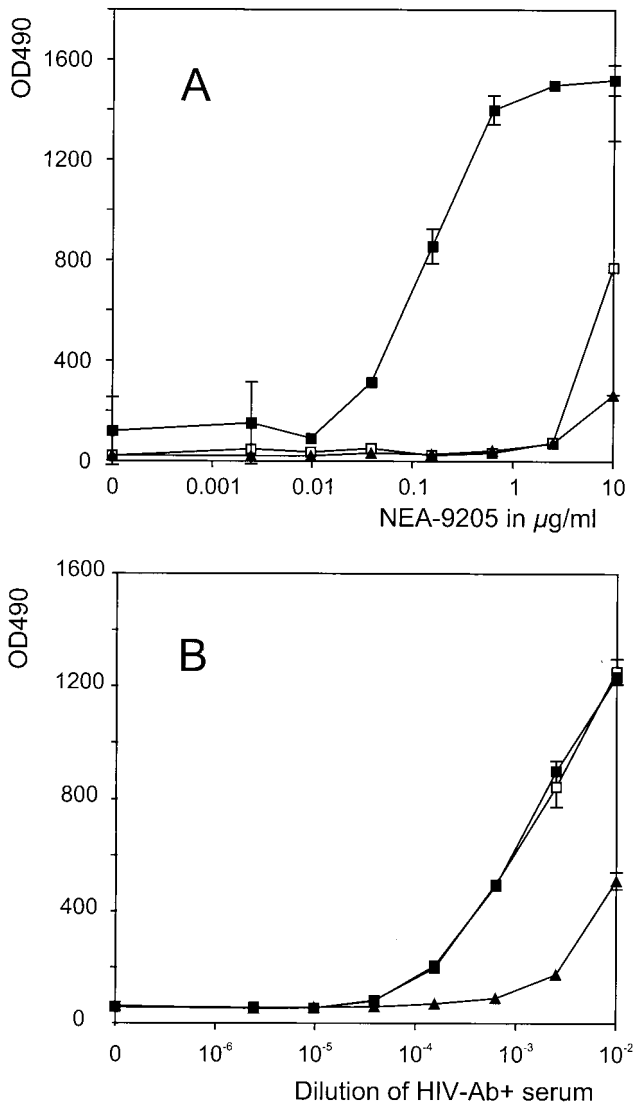

FIG. 1. Binding of NEA-9205 to immobilized gp120 from HIV-A308 and HIV-A308T321. gp120 from HIV-A308 (solid squares) and HIV-A308T321 (open squares) was captured to a solid phase by D7324 and subsequently detected with NEA-9205 (A) or serum from an HIV-infected individual (B). The background signal, i.e., the signal obtained without antigen captured to the solid phase, is shown for each dilution (triangles). The standard deviation of duplicate determinations is indicated by error bars or is within the symbol. NEA-9205 bound HIV-A308 with high affinity in contrast to HIV-A308T321 gp120, which was resistant to binding (A) when amounts of antigen isoreactive with serum from a HIV-infected individual were loaded to the solid phase (B). OD_{490}, optical density at 490 nm.

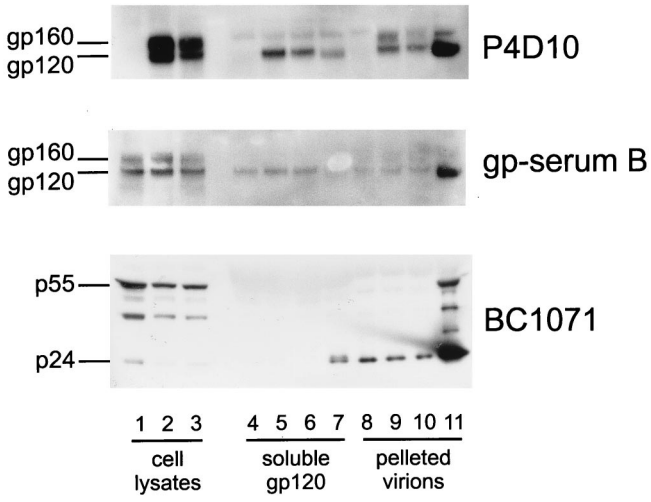

FIG. 2. Coexpression of envelope-genes. HEK293 cells were transfected with pHxBΔCat and pSV-A308T321 (lanes 1, 4, and 8), -A308 (lanes 2, 5, 9) and an equal mixture of the two (lanes 3, 6, and 10). Cell lysates (lanes 1 to 3), virion-depleted supernatants (lanes 4 to 6), and virion pellets (lanes 8 to 10) were prepared 72 h posttransfection and subjected to immunoblotting using as indicated MAb P4D10 specific for HIV-A308 gp120, immune guinea pig serum gp-serum B recognizing both HIV-A308 and HIV-A308T321 gp120, and MAb BC1071 against Gag proteins as detecting reagents. For comparison, a virion-depleted supernatant (lane 7) and a virion pellet (lane 11) derived from a fully infectious HIV-A308 clone propagated in H9 cells were loaded onto the gel. The expression of Envelope protein on the virion was regulated by varying the relative proportion of pSV-A308 to pSV-T321 in the transfection mixture.

could be controlled by adjusting the amounts of transfected Envelope-expressing plasmids. Thus, by variation of the ratio of pSV-A308 to pSV-A308T321 in the cotransfection mixture, the proportion of MAb binding sites on virions generated by *env* transcomplementation could be regulated.

To compare the composition of virions generated by *env* transcomplementation with the composition of T-cell line-adapted virus propagated in $CD4^+$ cells, a preparation of a fully replicating clone of HIV-A308 propagated in H9 cells was included on the blot (Fig. 2, lanes 7 and 11). As shown, virion pellets prepared from the supernatants of infected H9 cells contained more Env protein than did virion pellets generated by *env* transcomplementation. In contrast, the virion-depleted supernatant from HEK293 cells contained significantly more soluble gp120 than did the supernatant from H9 cells. Significantly more gp160 than gp120 was pelleted from HEK293-generated supernatants than from supernatants generated from H9 cells. Whether gp160 is incorporated into the virions or is present in cellular vesicles pelleted during centrifugation is presently unknown. The increased levels of soluble gp120 and pelleted gp160 in preparations generated from *env*-transcomplemented HEK293 cells compared to HIV-infected H9 cells may reflect an increase in Env to Gag expression in *env*-transcomplemented cells compared to HIV-infected cells. However, this issue is not addressed in Fig. 2. To assess the number of virions present in each preparation of pelleted virions, the blot was reprobed with MAb BC1071 specific for p24. Although the loading of the blot is unbalanced, densitometric analysis confirmed that the Env-to-Gag ratio on virions generated by *env* transcomplementation was not decreased compared to that on virions prepared from infected H9 cells. Thus, virions prepared by *env* transcomplementation may be expected to be neutralized similarly to virus propagated in $CD4^+$ cells.

Neutralization of chimeric virions. Hypotheses regarding the nature of MAb neutralization include the neutral hypothesis (each gp120 molecule contributing equally to the infectivity of the virus), the single-hit hypothesis (a single bound antibody neutralizing the infectivity of the virion), the multiple-hit/threshold hypothesis (the neutralizing of the infectivity when a critical amount of antibody is bound to the virion), and the complete-occupancy hypothesis (the neutralizing of the virion when all binding sites for the antibody are occupied). How chimeric virions in which the proportion of binding sites for the neutralizing antibody is varied is expected to be neutralized according to each of the hypothesis above is shown in Fig. 3. As shown, if neutralization is single hit, the infectivity of

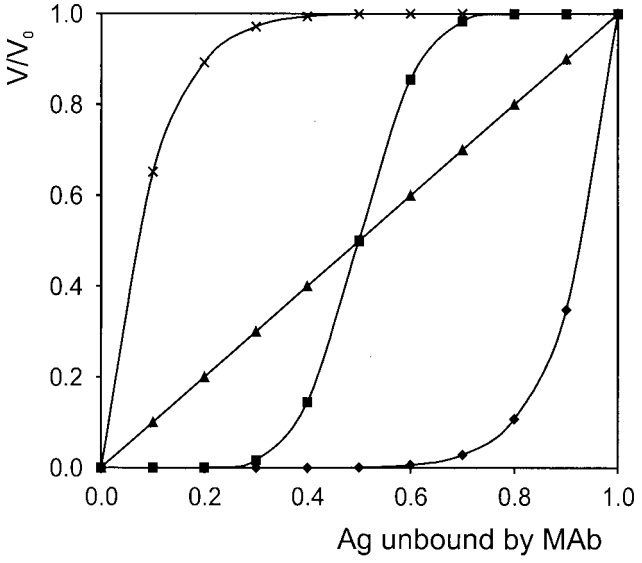

FIG. 3. Stoichiometry of MAb neutralization. The level of infectivity expected to survive MAb neutralization under different assumptions for the stoichiometry of MAb neutralization is shown as a function of the proportion of antigen (Ag) unbound by MAb. If each gp120 molecule contributes equally to the infectivity of the virion, the infectivity of the surviving MAb neutralization can be expected to be linearly dependent on the proportion of Ag unbound by MAb (triangles). If a single MAb bound to a virion neutralizes the virion, infectivity surviving neutralization can be expected to decrease abruptly as some antigen is bound by MAb (diamonds). If all available sites are occupied by MAb before the virion is neutralized, infectivity is unaffected by MAb binding until a large proportion of Ag is occupied (crosses). If neutralization occurs through MAb binding by multiple antibodies reaching the threshold required for neutralization, infectivity surviving neutralization can be expected to decrease abruptly when the threshold has been exceeded (squares).

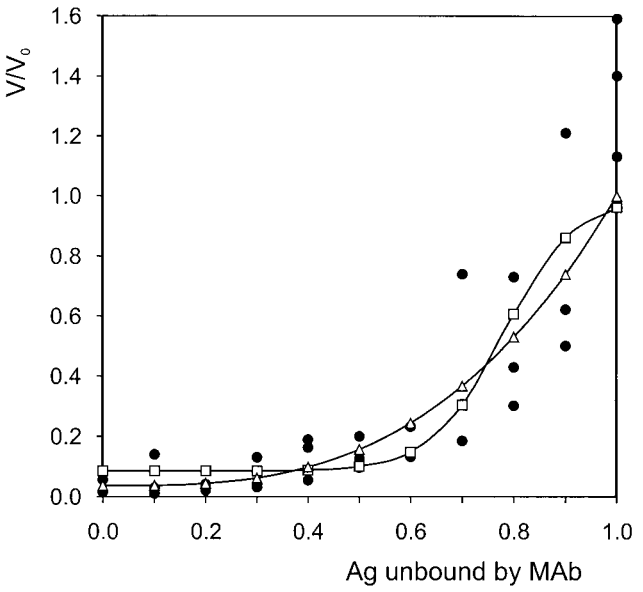

FIG. 4. Neutralization of Env chimeric virions. Virus supernatants produced by *env* transcomplementation with A308-*env* and A308T321-*env* were neutralized by using NEA-9205 at 2 μg/ml. Infectivity surviving neutralization (V/V_0) is shown as a function of the amount of antigen (Ag) unbound by MAb present, i.e., the proportion of A308T321 used for *env* transcomplementation. The results of three independent experiments are shown (filled circles). Nonlinear regression was performed, fitting the experimental data either to a third-order power function (open triangles; equation 2 in the text) or to a sigmoid function (open squares; equation 3 in the text). The functions fit the experimental data with comparable accuracy.

chimeric virus is expected to be neutralized as soon as only a few antibody binding sites remain on the virions. In contrast, if neutralization occurs only after all binding sites are occupied, the presence of Envelope molecules resistant to MAb binding is expected to result in full infectivity of the virions. If neutralization occurs by a multiple-hit/threshold mechanism, the expected neutralization curve will be in between the two aforementioned extremes and will be characterized by a lag phase at which the fraction of MAb binding sites reaches a critical threshold where infectivity in the presence of MAb is affected. If each Envelope molecule contributes equally to the infectivity of the virion suspension, then infectivity in the presence of MAb can be expected to change in proportion to the amount of Envelope resistant to MAb binding present in the virions.

Neutralization of chimeric virions generated by coexpression of pSV-A308 and pSV-A308T321 in an *env* transcomplementation assay was done by using NEA-9205 at a concentration of 2 μg/ml. The total amount of *env*-expressing plasmids was kept constant in each transfection, whereas the fraction of pSV-A308T321 was varied between 0 and 100% in increments of 10%. The fraction of virus surviving MAb neutralization as a function of the content of pSV-A308T321 in the transfection mixture of three independent experiments is shown in Fig. 4. Several conclusions can be reached on the basis of this result. First of all, as the number of binding sites for the neutralizing MAb increased (i.e., as the amount of pSV-A308T321 transfected decreased and the amount of pSV-A308 increased), virus rapidly became susceptible to neutralization, excluding the possibility of a complete-occupancy mechanism of MAb neutralization. Additionally, as the available binding sites increased, virus retained some infectivity that survived MAb neutralization, rendering single-hit neutralization of virion infectivity unlikely. Rather, binding of antibody to virions affected infectivity quantitatively in a nonlinear manner. This may suggest that the functional unit neutralized by MAb binding and contributing to the probability of successful infection is smaller than the virion and larger than the gp120 molecule itself.

Neutralization through MAb-induced aggregation of virions was not expected to contribute significantly to the neutralization observed, as the assay was done with excess antibodies and the virion suspensions produced by Env transcomplementation contained considerable amounts of shed gp120 (Fig. 2). To fully exclude the possibility that neutralization through virion aggregation occurred in a significant degree, pseudotypic virions containing AMLV Env and HIV-A308 Env were produced. HIV-A308 and AMLV Env cannot be expected to form mixed oligomers. NEA-9205 significantly neutralized infection of $CD4^+$ cells by A308/AMLV chimeric virions; however, the antibody did not neutralize infection of CD4- HEK293 cells by the same virus supernatant (Table 1). Thus, virion aggregation induced by MAb binding of HIV-A308 Env did not occur to an extent that interfered with AMLV Env function.

Neutralization was for all fractions of pSV-A308 transfected greater than the proportion of MAb binding sites available (cf. Fig. 3 and 4). The fact that MAb neutralization was progressive relative to MAb binding suggested that the functional infectious unit of the virion was larger than the Envelope molecule itself. At the concentration of MAb employed, a small fraction of a pure pSV-A308-complemented virus preparation remained unneutralized. To obtain a more precise description of the relationship between the proportion of Envelope antigen

TABLE 1. Neutralization of HIV(AMLV Env/A308 Env) pseudotypes

Pseudotyped Envelope[a]	Target cell	NEA-9205 (2 μg/ml)	CAT activity[b] (10^3 cpm)
A308/AMLV	HeLa-CD4	−	13
		+	3.1
	HEK293	−	2.9
		+	4.5
AMLV	HeLa-CD4	−	38
		+	43
	HEK293	−	21
		+	31
A308	HeLa-CD4	−	34
		+	0.9

[a] If more than one Envelope is indicated, virions were generated by transcomplementation, with a 1:1 ratio of the Envelopes indicated.
[b] Average of duplicate wells.

TABLE 2. Neutralization of A308T321/A308K373 pseudotypes

Pseudotyped Envelope[a]	NEA-9205 (2 μg/ml)	CAT activity[b] (10^3 cpm)	+MAb/−MAb
A308T321/A308	−	53 ± 8.7 (1.00)	
	+	7.0 ± 0.8 (0.13)	0.13
A308T321/A308K373	−	16 ± 1.0 (0.31)	
	+	7.5 ± 0.5 (0.14)	0.46
A308K373	−	0.036 ± 0.012 (0.00)	

[a] If more than one Envelope is indicated, virions were generated by transcomplemention with a 1:1 ratio of the Envelopes indicated.
[b] Average of quadruplicate wells with standard errors of the means indicated. Values normalized to A308T321/A308-complemented supernatants in the absence of MAb are shown in parentheses.

resistant to MAb binding and infectivity surviving MAb neutralization, nonlinear regression analysis was performed. In the experiments reported, infectivity remaining after neutralization of pSV-A308T321-complemented virus was, on average, 1.37. This apparent enhancement was not reproduced in further experiments with a dilution series of the MAb (data not shown) and was not previously observed with the same MAb and virions produced in H9 cells (31). Additionally, at the MAb concentration used in the present study, we were unable to demonstrate specific binding of the MAb to A308T321 gp120 (Fig. 1). For these reasons, nonlinear regression analysis was performed by using a theoretical value of maximum infectivity surviving neutralization on 1.0 and allowing for the existence of an unneutralized fraction. Initial analysis suggested that the experimental data could be fit to a power function as follows:

$$V/V_0 = (1 - \mathrm{Uf}) \times (A\mathrm{g}_{\mathrm{res}})^n + \mathrm{Uf} \quad (1)$$

where V/V_0 is infectivity surviving neutralization, $\mathrm{Ag}_{\mathrm{res}}$ is the proportion of Envelope antigen resistant to MAb binding, and Uf is the unneutralized fraction. Nonlinear regression was performed to obtain an estimate of the exponent n. The best fit was obtained for $n = 3.13$ (±0.68) (mean ± standard deviation). If the assembly of Env oligomers is based on random recruitment from a common pool of subunits, then the term $(\mathrm{Ag}_{\mathrm{res}})^3$ signifies the proportion of Env trimers consisting exclusively of A308T321 Ag and, therefore, the proportion of Env trimers incapable of binding the neutralizing MAb. Regression analysis was therefore redone for $n = 3$, and the following equation was obtained:

$$V/V_0 = (1 - 0.04) \times (\mathrm{Ag}_{\mathrm{res}})^3 + 0.04 \; (R^2 = 0.8031) \quad (2)$$

This equation, shown in Fig. 4 (triangles), underscores the cooperativity of MAb neutralization and is fully compatible with a model of the Envelope oligomer as the minimal functional unit neutralized by MAb binding. This model did not differ significantly from the experimental data as evaluated by the runs test ($P = 0.46$).

The apparent absence of a lag phase before the beginning of neutralization seems to argue against a multiple-hit/threshold model for neutralization. For a more stringent test of this hypothesis, the experimental data were fit to a sigmoid curve. A maximum V/V_0 of 1.0 yields the following fit:

$$V/V_0 = 0.08 + 0.92/(1 + e^{14.1(0.78-\mathrm{Ag}_{\mathrm{res}})} \; (R^2 = 0.7736) \quad (3)$$

This may be interpreted as a model for neutralization in which the virion is neutralized when a threshold has been reached. The threshold is defined by the value for which the exponent in equation 3 is zero; i.e., for the best fit for $\mathrm{Ag}_{\mathrm{res}} = 0.78$ (±0.03). Neither this model differs from the experimental data as evaluated by the runs test ($P = 0.41$). Thus, the lack of an observable critical threshold in Fig. 4 does not exclude the possibility that MAb neutralization occurs by a multiple-hit/threshold mechanism.

Neutralization through MAb binding to functionally defective Envelope. A previously described mutation, D373K, in HIV-BRU gp120 disrupts CD4 binding (25) and acts as a transdominant negative mutant (19). Virus was generated by *env* transcomplementation by cotransfection of equal amounts of pSV-A308 and pSV-A308T321, pSV-A308T321, and pSV-A308K373 and by transfection of pSV-A308K373 alone. The results are shown in Table 2. As expected, A308K373-Envelope-complemented virus showed infectivity levels only marginally above assay background levels. The infectivity of a A308T321/A308K373-complemented supernatant was 31% of that of a A308T321/A308-complemented supernatant, confirming the transdominant negative potential of the D373K mutation in the genetic context of the T308A mutation. More importantly, however, MAb binding to A308K373 Envelope in a A308K373/A308T321-complemented supernatant was neutralizing, reducing the infectivity from 31 to 14% of a A308T321/A308-complemented supernatant. This may indicate that mixed A308K373/A308T321 trimers retain residual function and that this function is neutralized by MAb binding. The infectivity remaining after neutralization was in close agreement with the infectivity remaining in a A308T321/A308-complemented supernatant after neutralization (13%) which, in turn, is close to what can be expected based on equation 2. Furthermore, A308T321/A308K373 pseudotyped virions were less sensitive to MAb neutralization than A308T321/A308 pseudotyped virions (53 versus 87% neutralization) (Table 2). The reduced sensitivity of A308T321/A308K373 pseudotyped virions compared to that of A308T321/A308 pseudotyped virions may reflect the reduced functionality of A308T321/A308K373 mixed trimers compared to that of A308T321/A308 mixed trimers. Thus, the experimental data obtained are fully compatible with incremental MAb neutralization and with the occurrence of MAb neutralization through binding to a defective Env molecule by neutralization of the residual function of the mixed trimer.

DISCUSSION

In the present study, we investigated the MAb neutralization of virions generated by *env* complementation coexpressing a neutralization-sensitive Envelope binding the neutralizing MAb and a homologous, neutralization-resistant Envelope not binding the neutralizing MAb. The results obtained support a model for progressive, incremental MAb neutralization of the virion as each Envelope oligomer binds a single MAb. In this sense, each virion contains multiple Envelope oligomers whose function can be neutralized independently of each other. Conversely, each Envelope oligomer may contribute independently to the likelihood of infection. In assays determining the kinetics of antibody neutralization, incremental neutralization is expected to display apparent first-order kinetics (16). Thus, incremental neutralization is consistent with experimental data from previous reports demonstrating apparent first-order kinetics of HIV neutralization (22).

The experimental data on neutralization of chimeric viruses (Fig. 4) did not exclude the possibility that neutralization occurred by a multiple-hit/threshold mechanism with a threshold when about 20% of the antigenic sites are occupied by MAb and a lag phase before neutralization commences obscured by virion heterogeneity. The definitive assessment of the applicability of a threshold model awaits the development of an experimental system by which virion Env/Gag ratios can be regulated and measured.

The methodology employed in the present study and the proposed model for incremental MAb neutralization may be useful for studying intraoligomeric interactions. Here we show that MAb binding to a transdominant negative Envelope molecule defective in CD4 binding may neutralize Envelope function of chimeric virions. The obvious interpretation for this in the context of the proposed model for MAb neutralization is that mixed oligomers containing the transdominant negative mutant retain residual function. One implication of this interpretation is that defective Envelope molecules may be able to functionally complement each other within the oligomer. This has been documented both for MLV (27, 40) and HIV (28). However, these results may not be easily interpreted in the context of a threshold hypothesis.

Whether our results can be extended to antibodies with specificities other than V3 must be determined experimentally. The methodology used in the present study can be easily adapted to other Env species and to other MAbs for which suitable escape mutants have been generated. However, most gp120-directed MAbs may have a similar mechanism of action when neutralizing T-cell line-adapted HIV-1. Ugolini et al. (37) clearly demonstrated that inhibition of attachment could account for a significant fraction of the neutralizing effect of gp120-directed antibodies. Furthermore, in a recent study, the ratio between virion MAb binding and neutralization was determined for a panel of HIV-1-neutralizing antibodies (26). This ratio was the same, within experimental error, for the MAbs tested, regardless of epitope specificity. Thus, the stoichiometry and mechanism of neutralization may be the same for all gp120-specific antibodies. Although our data include only a single MAb and a single T-cell line-adapted strain of HIV-1, these findings suggest that the conclusions could be extrapolated to other epitopes.

The extent to which the results may be extended to primary isolates of HIV-1 remains uncertain. Primary isolates are generally more resistant to antibody neutralization than isolates adapted to growth in T-cell lines (24). Furthermore, primary isolates have a higher Env/Gag ratio (spike density) than T-cell line-adapted isolates (34). In a mathematical model of antibody neutralization, it was proposed that Env spike density could be an important modifier of sensitivity to antibody neutralization if this occurred through an occupancy/threshold mechanism, perhaps explaining the relative neutralization resistance of primary isolates of HIV (18). The suggestion that MAb neutralization is incremental does not support the role of Env spike density as a primary modifier of neutralization susceptibility and resistance. Likewise, it was subsequently demonstrated that neither increased Env spike density nor Env stability is required for neutralization resistance of primary isolates (17); rather, the neutralization resistance of primary isolates can be attributed at least in part, to a lower affinity of antibody to primary Env trimers compared with antibody to T-cell-line-adapted Env trimers (29). An additional explanation of the relative neutralization resistance of primary isolates may be that the outcome of the interaction between primary isolate and antibody is fundamentally different from the outcome of the interaction between T-cell line-adapted virus and antibody. Recent data demonstrate that some primary isolates are activated by antibody binding rather than being neutralized (32, 33, 35). Our results as well as the majority of work to elucidate mechanism and stoichiometry of HIV-1 neutralization have been done with T-cell line-adapted isolates of HIV-1 (26, 37) and should not readily be extended to primary isolates of HIV.

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